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Effect of Dengue Virus on Procoagulant and Fibrinolytic Activities of Monocytes

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Some of the fibrinolytic and coagulation enzymes that monocytes produce are urokinase, a plasminogen activator (PA); a PA-specific inhibitor (PAI); and procoagulant activity (PCA), which has been characterized as tissue factor. Dengue infection *in vivo* is restricted to monocytes; however, it is unknown if dengue-infected monocytes undergo alterations in the production of PA, PAI, and PCA. This issue was addressed in studies in which monocytes were infected *in vitro* with dengue 2 virus in serum-free medium in the presence of enhancing antibody. No urokinase activity was detected in either control or infected cells or in their supernatants. Infection of monocytes with dengue 2 virus resulted in an almost threefold increase in PAI activity in cells and supernatants. No change in relation to the control was observed in PCA generated by the infected cells. These data indicate that dengue 2 infection enhances the production of PAI from monocytes without altering PA or PCA.

Dengue viruses are flaviviruses that comprise four distinct types (dengue types 1–4). These viruses are transmitted by the domestic mosquito *Aedes aegypti* and cause disease with two different clinical manifestations, one mild, i.e., dengue fever (DF); and the other life-threatening, leading to shock and known as dengue shock syndrome or dengue hemorrhagic fever (DSS/DHF). DF is typically seen in adults, whereas DHF/DSS occurs predominantly in children [1].

Disseminated intravascular coagulation appears to be responsible for the hemorrhagic tendencies seen in patients with DHF [2]. In some situations, however, deposition of fibrin has also been observed. In an epidemic of Philippine hemorrhagic fever in 1956, the virus was identified as dengue 3 and intravascular thrombosis was observed in 12 patients, five of whom had arteriolar thrombosis in the renal glomeruli [3]. Srichaikul et al. studied three Thai men who presented with profound shock and gastrointestinal bleeding [4]. Two of the patients died; at autopsy fibrin thrombi were found. However, the pathogenesis of the thrombosis was not clearly defined in that report and remains elusive. The increased localized deposition of fibrin could be due to increased levels of inhibitors of the fibrinolytic system. These inhibitors are produced either by endothelial cells (known

as plasminogen activator inhibitor 1 [PAI-1]) or by monocytes (PAI-2). Levels of these inhibitors have not been measured in plasma from patients or in monocytes infected *in vitro* with dengue virus.

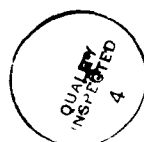
Site of Infection and Immune Enhancement

Attempts have been made to identify the site of proliferation of dengue virus in humans. Dengue viruses have been recovered from peripheral blood leukocytes (probably monocytes) from children with DHF/DSS [5]. In another study [6] fluorescein-conjugated antibodies to dengue virus were used to demonstrate the presence of virus in monocytes obtained from children infected with dengue. Available evidence suggests that dengue 2 infection *in vivo* is possibly restricted to cells of mononuclear lineage. Dengue virus has been successfully replicated in monocytes *in vitro* [7, 8]. There has been only one report of the growth of dengue 2 virus in human endothelial cells *in vitro* [9]; however, demonstration of infection of endothelial cells *in vivo* in children with DHF/DSS has not been successful [10].

One of the immunopathologic mechanisms attributed to dengue 2 is the ability of antibody to mediate infection in monocytes. Human cord blood containing antibody to dengue in appropriate titers can enhance dengue infection in vitro and in vivo. This phenomenon allows an understanding of the age distribution among infants with DHF/DSS [1]. During the first few months after birth, the titers of maternal antibody are sufficiently high to protect the infant from dengue. With time, the levels of antibody

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decrease and can then act as mediators or enhancers of dengue infection, thereby causing infants <1 year of age to develop the shock syndrome. When the infant is about a year old, the levels of antibody are negligible and the infant is no longer at risk for DSS.

When dengue 2 antibody is added to cultures of peripheral blood leukocytes at concentrations below those that neutralize the viral inoculum, an antibody-virus complex is formed and then ingested by the cell [8]. Antibody-enhanced infection in bone marrow cultures also appears to involve mononuclear phagocytes [7]. Halstead et al. reported that enhancement of dengue 2 infection in human peripheral blood leukocytes *in vitro* is a property of antisera raised to any flavivirus [11]. Thus, humans exposed to non-dengue flaviviruses may become more susceptible to dengue virus.

Procoagulant and Fibrinolytic Activity of Monocytes

Human peripheral blood mononuclear cells generate procoagulant activity that initiates the extrinsic cascade of blood coagulation and has been identified as tissue factor by immunologic characterization [12]. When monocytes are exposed to exogenous agents such as phytohemagglutinin [12] or endotoxin [13, 14] or undergo adherence to glass [15], this activity increases. Direct interaction between lymphocytes and monocytes appears to be essential for the induction of PCA. Levy et al. have shown that when T lymphocytes are stimulated by endotoxin and then exposed to monocytes maximal induction of PCA

occurs. Triggering of the monocytes by the stimulated lymphocytes is not dependent on protein synthesis, whereas the generation of PCA by monocytes is sensitive to inhibition by both cycloheximide and actinomycin D. Thus, generation of PCA requires *de novo* synthesis of both RNA and protein [13].

As illustrated in figure 1, tissue factor acts through the extrinsic pathway in the coagulation cascade. It activates factors VII to VIIa, and factor VIIa then activates factors X to Xa. Factor Xa—along with factor V, Ca^{++} , and phospholipid—activates factor II to IIa, which in turn converts fibrinogen to fibrin and forms a clot. While tissue factor acts via the extrinsic pathway, factors XII, XI, IX, and VIII act through the intrinsic pathway.

In addition to PCA, monocytes produce urokinase, a plasminogen activator (molecular weight, 55,000). Urokinase converts plasminogen to plasmin, which in turn lyses fibrin clots [16, 17]. Monocytes also produce a PAI-2 (molecular weight, 47,000), a fast-acting, specific inhibitor of urokinase [18].

Effect of Dengue Infection on Urokinase, PAI, and PCA in Monocytes

It has been reported that monocytes produce urokinase, PAI, and PCA, but it is not known if dengue infection alters the expression or production of these proteins. Previous work has shown that synthesis of PAI-2 can be stimulated by exposing monocytes to endotoxin or to phorbol ester, a tumor-promoting agent [18, 19]. Thus, the following experiments were

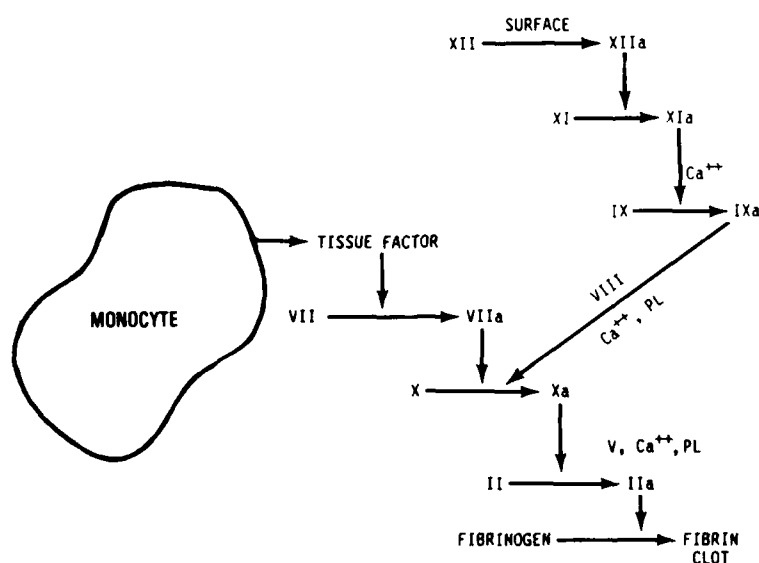


Figure 1. A schematic diagram of the coagulation cascade. Monocytes produce tissue factor, which initiates clot formation via the extrinsic pathway by activating factor VII. Factors XII, XI, IX, and VIII mediate formation of fibrin via the "intrinsic pathway." The activated forms of the factors are represented by the suffix "a." Ca^{++} and phospholipid (PL) are required for the activation steps indicated.

conducted to determine if dengue virus altered these procoagulant and fibrinolytic activities.

Materials and Methods

Monocytes were obtained by countercentrifugal elutriation and infected with dengue virus in serum-free RPMI medium containing 0.6% bovine serum albumin in polypropylene tubes. Cells were infected with dengue 2 virus (strain, PUO-218) obtained from a Thai child with primary DF. The multiplicity of infection (MOI) was 0.5, and incubation was carried out in the presence of enhancing antibody (mouse hyperimmune ascitic fluid) at a dilution of 1:5,000. Control cultures were treated with the same dilution of enhancing antibody. On day 3 or 4, cells were counted and viability was assessed by exclusion with trypan blue. In all the experiments, viability was >99% until day 4. Supernatants were collected after centrifugation, aliquoted, and frozen at -70°C until assays for urokinase and PAI could be performed. Cells were washed, resuspended, aliquoted, and frozen until assays for urokinase, PAI, and PCA could be conducted.

PCA was assessed by the following method: cells were thawed and homogenized, and recalcification times were assessed with use of normal and factor-deficient plasmas; 0.1 mL of plasma was preincubated with a source of tissue factor at 37°C for 2 minutes, and then 0.1 mL of CaCl_2 (0.025 M) was added. The clotting time was recorded on a Coagulation Profiler (Bio Data Corporation, Model CP-8, Horsham, Pa.).

Assays for urokinase and PAI were conducted according to the method of Chmielewska et al. [20]. In brief, assays for urokinase involved the incubation of samples with plasminogen and the chromogen S-2251 at 37°C in a microtiter plate. The char-

acter in absorbance was measured at 405 nm with a Titer-tek Multiscan Spectrophotometer (Flow Laboratories, McLean, Va.). Activities were compared with that of a urokinase standard. PAI activity was assayed by preincubating samples with 50 mU of urokinase for 30 minutes at 22°C . Plasminogen and S-2251 were then added, and residual urokinase activity was measured after subsequent incubation at 37°C (1 mU of PAI is defined as the amount that inactivates 1 mU of urokinase during 30 minutes of incubation at 22°C ; PAI is expressed as mU/ 10^6 cells).

Results and Discussion

The effect of infection with dengue 2 on PCA in monocytes was determined by comparing the recalcification times of infected and noninfected cells (table 1). Normal pooled human plasma incubated with medium in the absence of cells had a recalcification time of 121 seconds. When control cells were incubated with normal plasma, clotting time was shortened to 82 seconds. This result was similar to the clotting time of 78 seconds obtained for the infected cells. These results appeared to show the presence of PCA, though no marked differences were seen between the infected and the noninfected cells. This PCA was shown to be tissue factor by incubation with factor-deficient plasmas. In comparison with the factor-deficient plasmas incubated with medium alone, the infected and control cells exhibited a greater shortening of recalcification time in the case of the factors VIII and IX as compared with factor VII- and factor X-deficient plasmas. This was expected, since tissue factor acts through the extrinsic pathway, and shortening of recalcification time could still occur in plasmas deficient only in factors of the intrinsic pathway (i.e., factors VIII and IX). Recalcification times were similar when infected or nonin-

Table 1. Recalcification times of normal and factor-deficient plasmas in the presence of noninfected and dengue-infected human monocytes.

Source of tissue factor	Recalcification time of indicated plasma				
	Normal	Factor VII	Factor X	Factor VIII	Factor IX
Medium	121 \pm 1	143 \pm 2	>500	>500	>500
Noninfected monocytes	82 \pm 2	105 \pm 3	262 \pm 4	83 \pm 2	100 \pm 1
Dengue-infected monocytes	78 \pm 2	101 \pm 2	247 \pm 6	72 \pm 1	86 \pm 3

NOTE. Day-4 cultures were used as the source of tissue factor. Values are the mean \pm SE of triplicate or quadruplicate determinations. See Materials and Methods for description of method used for determining recalcification times.

Table 2. Effect of dengue infection on production of plasminogen activator-specific inhibitor (PAI) activity by monocytes.

Monocytes	PAI activity (mU/10 ⁶ cells)	
	Cell-associated	Supernatant
Noninfected	148 ± 8	118 ± 15
Dengue-infected	374 ± 7	257 ± 11

NOTE. PAI activity was determined according to the method of Chmielewska et al. [20]. The values are the mean ± SE of triplicate determinations. See Materials and Methods for description of assay.

ected cells were incubated with plasmas deficient in factors VII, X, VIII, and IX.

PCA can be stimulated in response to viruses in monocytes, although optimal production requires the collaboration of T lymphocytes [13]. Since our experiments were conducted with a 99% pure population of monocytes, the maximal expression of PCA may not have been induced. Dengue 2 infection did not enhance production of PCA, when compared with that of the noninfected cells.

No urokinase activity was detected in either the control or infected cells or in their supernatants. Results of assays for PAI indicated the presence of an inhibitor in both control and infected cells and in their supernatants. Initial increases in PAI activity were detected as early as 24 hours after infection. By 3 days, when viral titers had reached their peak, PAI activity in the supernatants and cells was at least twofold higher in cultures infected with dengue 2 virus than in the controls (table 2). By day 6, cell-associated PAI activity had decreased while PAI activity in the supernatant had increased, a finding that suggests the release of PAI into the supernatant (data not shown). The precise role of this increased production of PAI in dengue infection or in any other viral hemorrhagic infection remains to be established. The change in PAI activity may be sufficient to enhance clot formation at a localized site of inflammation.

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